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Induction of angiogenesis during the transition from hyperplasia to neoplasia

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It is now well established that unrestricted growth of tumours is dependent upon angiogenesis[1,2]. Previous studies on tumour growth, however, have not revealed when or how the transition to an angiogenic state occurs during early tumour development. The advent of transgenic mice carrying oncogenes that reproducibly elicit tumours of specific cell types[3–6] is providing a new format for studying multi-step tumorigenesis[7,8]. In one of these models, transgenic mice expressing an oncogene in the β-cells of the pancreatic islets heritably recapitulate a progression from normality to hyperplasia to neoplasia[6]. We report here that angiogenic activity first appears in a subset of hyperplastic islets before the onset of tumour formation. A novel *in vitro* assay confirms that hyperplasia *per se* does not obligate angiogenesis. Rather, a few hyperplastic islets become angiogenic *in vitro* at a time when such islets are neovascularized *in vivo* and at a frequency that correlates closely with subsequent tumour incidence. These findings suggest that induction of angiogenesis is an important step in carcinogenesis.

The insulin-producing β-cells are localized in approximately 400 focal endocrine islets embedded in the exocrine pancreas (Fig. 1*a*(i)). The neoplastic transformation of these cells has been accomplished through the use of a hybrid oncogene (*RIP-Tag*) that employs the rat insulin gene regulatory region to control expression of the SV40 large T oncoprotein[6]. We have studied one line of transgenic mice (RIP1-Tag2) in which virtually every β-cell already expresses the oncogene at birth[9]. Hyperplasia of β-cells first becomes apparent at 4–6 weeks of age, and by 9.5 weeks, 50% of the islets are composed of proliferating β-cells[10]. Yet only a few islets progress to histologically distinct carcinomas, which are present by 12 weeks of age in all mice of this lineage. Thus, it seems that oncogene activation and consequent β-cell hyperplasia are necessary, but not sufficient, to produce a carcinoma.

To determine when neovascularization was initiated during β-cell tumorigenesis, immunohistochemical studies were performed using anti-laminin antibodies. Laminin is a ubiquitous basement membrane component that serves as an excellent marker for developing capillaries within normal[11] and neoplastic[12] tissues. Normal pancreatic islets and early hyperplastic islets contain tubular capillaries that appear homogeneous in form (Fig. 1*a*(i)). A subset of the hyperplastic islets, however, exhibit capillaries of varying shape and size as well as altered basement-membrane-staining patterns (Fig. 1*a*(ii)) due to formation of new capillary sprouts (Fig. 1*b*). Similar analyses of solid tumours revealed many new capillaries as well as complete disorganization of islet architecture (Fig. 1*a*(iii)).

The onset of new capillary growth within transgenic islets was confirmed using ^{3}H-thymidine autoradiography. Normal islet capillaries did not incorporate thymidine during the labelling periods. This is consistent with previous reports of a very low ^{3}H-thymidine labelling index of 0.01% in the capillaries of the pancreas[13]. By contrast, endothelial cells that incorporated ^{3}H-thymidine could be detected within capillaries in a subset of hyperplastic islets and within every large carcinoma (Fig. 1*c*).

These histological analyses demonstrated that neovascularization is a quality of every solid tumour, but only of an occasional hyperplastic islet. The results imply that an angiogenic capability develops as a consequence of some localized change within the population of hyperplastic islets during the preneoplastic period. There are several alternative explanations: (1) Every hyperplastic islet composed of proliferating cells expressing the oncogene is angiogenic, but the surrounding environment of normal pancreas suppresses this intrinsic capability, a suppression from which an islet can sporadically escape; (2) when hyperplastic islets exceed a threshold size, angiogenesis is automatically elicited; or (3) there is an infrequent, positive switch to an angiogenic state by individual hyperplastic islets which is independent of size or surrounding environment.

To distinguish between these possibilities, we devised a novel *in vitro* assay to measure the response of capillary endothelial cells to individual hyperplastic nodules. Islets were removed from the pancreas by infusion of collagenase retrograde into the pancreatic ducts[14] and then purified on a Ficoll gradient. Neither normal nor hyperplastic islets were larger than 400 μm in diameter (data not shown). Therefore, a sieve of 560-μm mesh size was employed to separate hyperplastic islets from

TABLE 1 *In vitro* aniogenesis assays

Age (weeks)	Angiogenic islets/total no. islets analysed				No. solid tumours per mouse				
4	0/40	0/40	0/358*		0	0	0	0	0
5	0/66				0				
6	0/138	0/78	0/79		1	0	1		
7	1/101				0				
8	1/100				2				
9	5/132	3/118	3/40		4	7	8		
10	2/100	0/97			4	9			
11	3/88				3				
12	5/98	4/118	2/83	1/30	4	7	6	5	

Results of *in vitro* angiogenesis assays using islets isolated from 21 transgenic mice. Each ratio (angiogenic islets/number of islets) represents data from an individual mouse except where indicated. The number of solid tumours per mouse represents tumours retained by the 560-μm mesh during isolation of islets from a single pancreas.
* Combined data from 3 mice.

tumours. Tumours were then retrieved from the sieve and analysed separately. Individual islets or tumours were placed into the wells of 24-well plates, each of which contained capillary endothelial cells (70,000 ml^{-1}) in a solution of fibrillar collagen, which gels at 37 °C (Fig. 2). The behaviour of each islet and its surrounding capillary endothelial cells was observed over a 1–2 week period.

During the first day of incubation, endothelial cells anchored in the collagen and elongated in a random orientation, but did not proliferate or form capillary sprouts. In the presence of islets isolated from normal adult mice, endothelial cells remained in a random orientation with little or no locomotion or proliferation (Fig. 3*a*). By contrast, every solid tumour induced a dramatic radial alignment of endothelial cells within 24 hours in a pattern that converged toward the implanted tumour (Fig. 3*b*). This assay thus distinguishes the normal condition from the tumour that arises out of it, based on identification of angiogenic activity *in vitro*.

With the specificity of the assay established, islets were analysed from RIP1-Tag2 mice at weekly intervals (from 4–12 weeks of age). None of the islets from 4–5-week-old mice stimulated the capillary endothelial cells. A small subset of the islets isolated from older transgenic mice, however, exhibited clear angiogenic activity (Fig. 3*c*–*f*). The response of the endothelial cells to hyperplastic islets or to tumours recapitulated the known stages of neovascularization *in vivo*[15] and *in vitro*[16]. Namely, the endothelial cells aligned in a radial pattern (Fig. 3*d*), then migrated towards the islet (Fig. 3*e*) and formed endothelial cell sprouts and occasional capillary tubes (Fig. 3*f*).

The assay also revealed that induction of angiogenesis is a local event which is selective or specific for the activated islet(s). Often, when multiple islets were cultured in the same well, one islet elicited capillary ingrowth, whereas its neighbours did not (Fig. 3*c*). There was no correlation between the size of a hyperplastic islet and its ability to induce capillary ingrowth. In some cases, an islet was angiogenic immediately upon introduction into the gel, whereas in other cases angiogenic activity arose only after a period of incubation *in vitro*, ranging from 3–12 days. These results in conjunction with our *in vivo* studies confirm that onset of angiogenic activity occurs in hyperplastic islets before overt tumour formation.

The ability to isolate hundreds of individual islets at defined times during progression to neoplasia has allowed us to determine the angiogenic capacity of each population in a statistical fashion, and to relate it to the frequency with which focal hyperplasias progress to neoplasia.

In an analysis of 1,900 islets, the percentage of transgenic islets that elicited angiogenesis *in vitro* was 0% at 4–5 weeks; $0.57 \pm 0.35\%$ at 6–7 weeks; $2.8 \pm 0.6\%$ at 8–10 weeks; and $3.6 \pm 0.39\%$ at 11–12 weeks (Table 1). As shown in Fig. 4, there was

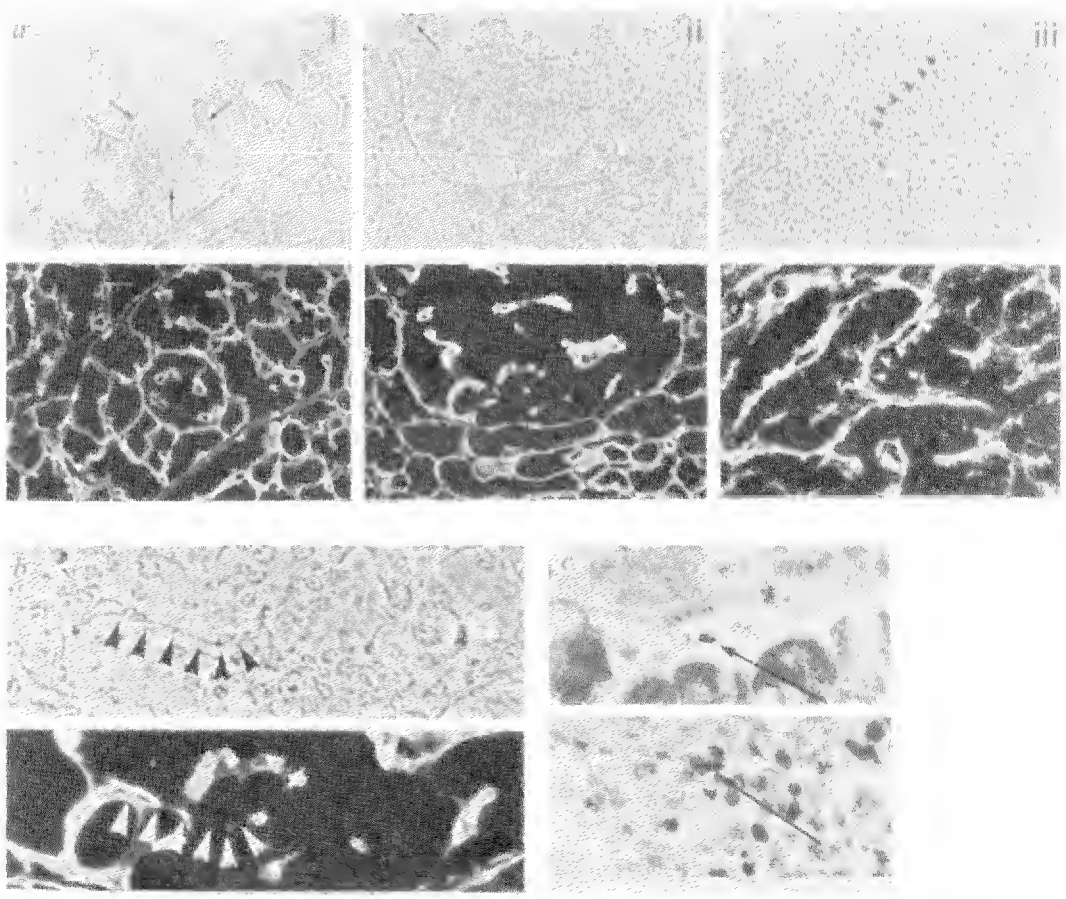

FIG. 1 Characterization of neovascularization *in vivo*. Phase contrast (upper panels) and corresponding immunofluorescence views (lower panels) of paraffin sections through a normal islet (*a*(i)), a hyperplastic transgenic islet (*a*(ii), *b*) and a transgenic islet cell tumour (*a*(iii)). *a*, (i) The normal islet of Langerhans (indicated by arrows) appears as a well-demarcated circular structure that is surrounded by clusters of exocrine acini. Laminin appears in a continuous pattern within all basement membranes. Islet capillaries appear as brightly stained tubular structures. *a*, (ii) This transgenic hyperplastic islet appears enlarged but continues to retain well-defined boundaries. The small arrow indicates a mitotic figure within the islet parenchyma. Laminin appears in a linear basement membrane pattern surrounding certain capillaries in this hyperplastic islet, although other more pleomorphic capillaries exhibit wispy and fibrillar laminin distributions. *b*, A higher magnification view of the same hyperplastic islet shows a capillary sprout branching from a pre-existing islet capillary. Large arrowheads point to a single capillary endothelial cell that stretches from the end of a lumenal cul-de-sac at the left and extends a long cell process to the right. The endothelial cell nucleus appears on the left between the first and second arrow-heads at the blunt end of the capillary lumen. Laminin appears in a linear pattern within the capillary basement membrane and in a fine, fibrillar distribution in association with the extended cell process. Note that the endothelial cell sprout and capillary containing a lumen both share a common basement membrane. *a*, (iii) Normal islet architecture has become radically disorganized and an extensive vascular network is evident within this islet cell tumour. The small arrowheads point to one tumour capillary. Laminin appears in both linear and diffuse, fibrillar patterns along tumour capillaries and connective tissue septae. Our method for immunostaining has been published previously[11]. *c*, ^{3}H-thymidine autoradiographs showing induction of capillary endothelial cell DNA synthesis *in vivo*. Large arrows point to endothelial cell nuclei that exhibit autoradiographic grains within capillaries in a hyperplastic islet (upper), and in the parenchyma of an islet cell tumour (lower). (Magnifications: *a*, ×140; *b*, ×350; *c*, ×450).

a striking correlation between the increasing incidence of transgenic islets that became angiogenic and the rising frequency of tumour formation. By contrast, the frequency with which hyperplastic islets developed was at all times much higher than the incidence of tumours. At 12 weeks, 75% of the islets were hyperplastic, whereas tumour incidence was only 1.5–2% (Fig. 4). Thus, it is the acquisition of angiogenic capacity by hyperplastic islets that scales with tumour incidence and not islet hyperplasia *per se*. These data show that induction of angiogenic activity and consequent neovascularization both precede tumour formation and correlate with the transition from hyperplasia to neoplasia.

This experimental model probably has an important counterpart in human cancer. Most cancers originate from epithelial cells, for example, carcinoma of the bladder, breast, cervix, colon and skin. A discernible pre-neoplastic stage lasting several

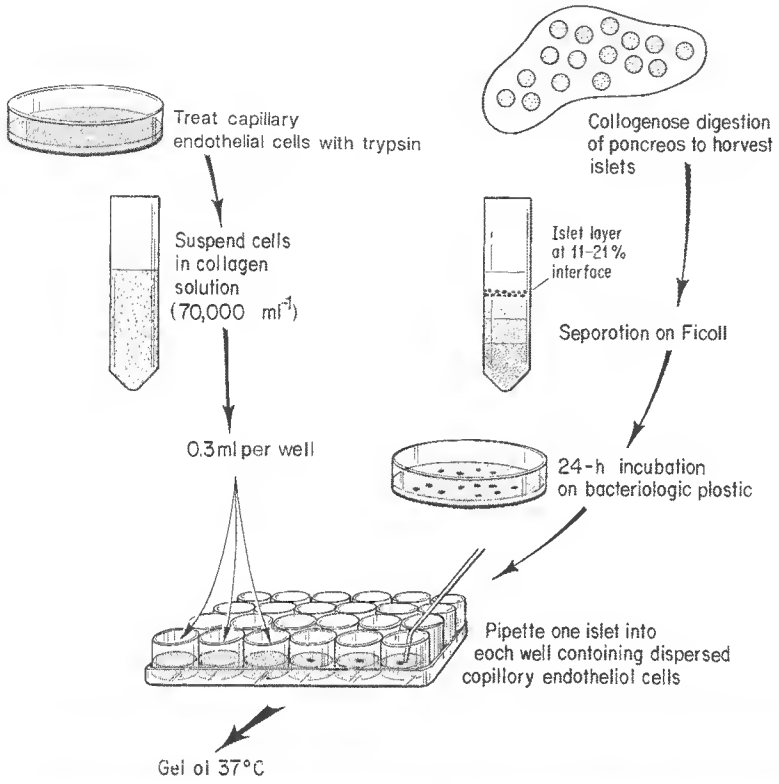

FIG. 2 *In vitro* gel assay for detection of angiogenic activity. Bovine capillary endothelial cells were cloned from adrenal cortex and passaged in gelatinized dishes as previously described[25]. For *in vitro* detection of angiogenic activity, endothelial cells were treated with trypsin and resuspended in a chilled collagen gel solution composed of Vitrogen (Collagen Corporation) mixed 1:1 with RPMI medium + 10% fetal bovine serum. Aliquots of the collagen-cell mixture were pipetted into 24-well tissue culture plates (Costar) and single pancreatic islets were added to each well before the solution was allowed to gel at 37 °C. Islets of Langerhans were harvested from mouse pancreases by intraductal injection of collagenase (Worthington IV CLS) as previously described[14]. Collagenase-digested pancreases were mechanically dissociated, passed through a stainless steel sieve (560 μm mesh), and islets were separated from other cells and debris using a Ficoll gradient (layers of 25%, 23%, 21% and 11% Ficoll). Islets were collected from the 21%/11% Ficoll interface and stored for 1–2 days at 37 °C in a bacteriological culture dish containing RPMI medium + 10% fetal bovine serum before use. Culture plates were incubated under 10% CO_2 and observed for endothelial cell reorientation, growth, migration, and differentiation (previous studies have shown that endothelial cells can form organized capillary tubes when grown on collagenous substrata[25–27]).

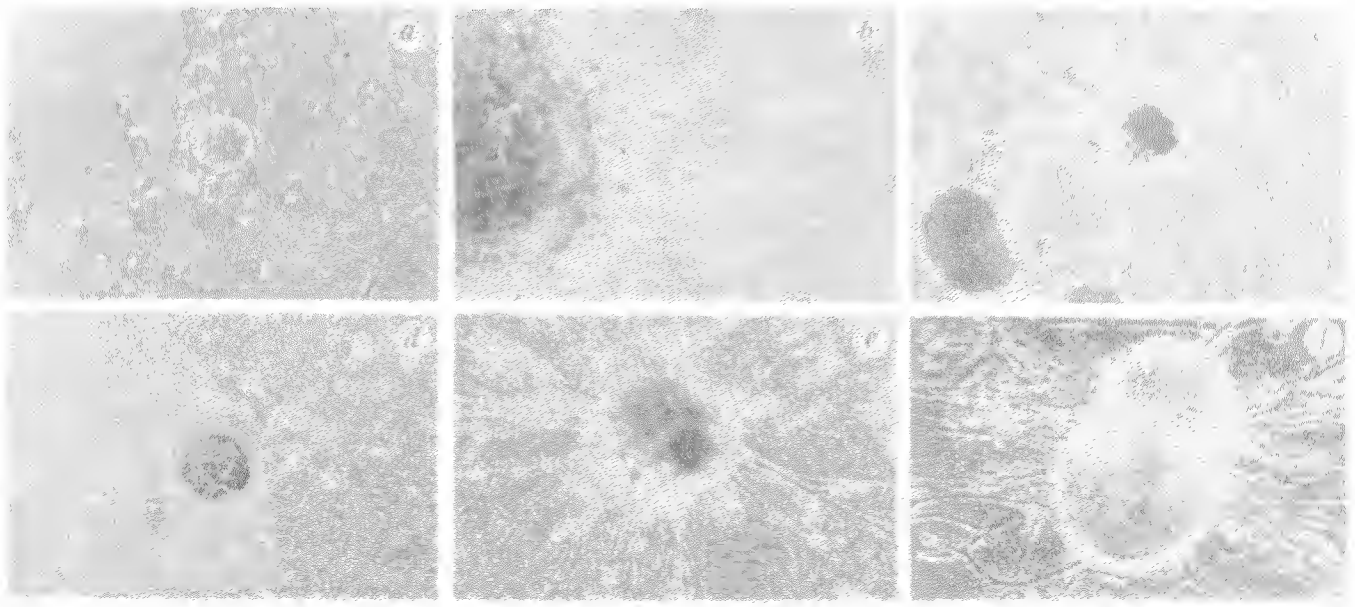

FIG. 3 *In vitro* assay of angiogenic activity during different stages in β-cell tumourigenesis. *a*, Normal islet from 14-week-old mouse exhibits a smooth border and is surrounded by randomly distributed capillary endothelial cells. *b*, Tumour from 12-week-old transgenic mouse induces extensive capillary endothelial cell ingrowth. *c*, 13-week-old hyperplastic islets. One islet in the centre is angiogenic, whereas the large islet at the bottom left is not. *d*, The initial response of endothelial cells to an angiogenic hyperplastic islet is realignment in a radial pattern. *e*, Alignment is followed by locomotion of endothelial cells and migration in tandem. The smooth border of the islet is destroyed when the migrating endothelial cells contact its surface (higher magnification of *c*). *f*, Migration of endothelial cells toward the angiogenic hyperplastic islet is followed by development of organized capillary networks. Magnification: *a–d*, ×47; *e*, *f*, ×93.

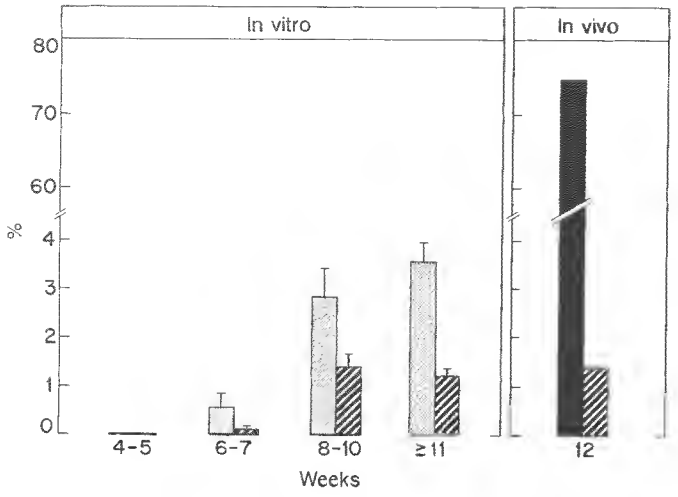

FIG. 4 Correlation of angiogenic activity with tumour incidence. The frequencies of angiogenic islets (stippled bars) and angiogenic tumours isolated *in vitro* (slashed bars, left panel) are compared with the incidence of hyperplastic islets (closed bar) and tumours *in vivo* (slashed bars, right panel). Tumours isolated *in vitro* are presented as a percentage of the approximate total number of islets present in each pancreas (400). The percentage of tumours present *in vivo* at 12 weeks is based upon routine autopsy of transgenic mice in this lineage. Islets are defined as hyperplastic *in vivo* if more than 20% of the β-cells incorporate ^{3}H-thymidine; the frequency of hyperplastic islets at 12 weeks is expressed as a percentage of total islets (from Teitelman *et al.*[10]).

years often precedes these malignancies[17–20]. A distinguishing feature of most pre-neoplastic lesions is their lack of obvious neovascularization, as compared with the resulting neoplasias which are typically highly angiogenic. The 'switch' from the pre-vascular state to the vascularized stage may be followed by an increase in growth rate and metastasis[21]. Analysis of this switch has been limited by the unavailability of reproducible samplings of pre-neoplastic lesions at defined times during their development. This realization motivated previous investigators to examine angiogenesis during *de novo* tumour formation in animal models[22–24]. Unfortunately, the irregularity of tumorigenesis has limited the use of this approach, particularly with regard to isolating and analysing the pre-neoplastic stages.

By contrast, pre-neoplastic islets appear in a predictable manner in transgenic mice and are accessible to study. The development of an *in vitro* bioassay detects precisely when these islets switch to the angiogenic state. In conjunction with *in vivo* analysis, this bioassay provides an unprecedented and powerful new method for future studies into the molecular genetic events that govern the induction of angiogenesis and tumour progression.

The consensus of these and other studies on tumorigenesis in transgenic mice is consistent with a model in which oncogenes abrogate growth control and induce cell proliferation and consequent hyperplasia. This abnormal cell proliferation then sets the stage for additional genetic or epigenetic secondary events. Induction of angiogenesis seems to be one of these events that is important for the conversion of normal epithelium into a cancer. □

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Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1

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THE leukocyte adhesion molecule LFA-1 mediates a wide range of lymphocyte, monocyte, natural killer cell, and granulocyte interactions with other cells in immunity and inflammation[1,2]. LFA-1 (CD11a/CD18) is a receptor for intercellular adhesion molecule 1 (ICAM-1, CD54), a surface molecule which is constitutively expressed on some tissues and induced on others in inflammation[3–5]. Induction of ICAM-1 on epithelial cells, endothelial cells and fibroblasts mediates LFA-1-dependent adhesion of lymphocytes[4,6,7]. Several lines of evidence have suggested the existence of a second LFA-1 ligand: homotypic adhesion of one cell line was inhibited by a monoclonal antibody to LFA-1, but not by one to ICAM-1[8]; there exists an LFA-1-dependent, ICAM-1-independent pathway of adhesion to endothelial cells[6]; and also, there are some types of target cells in which LFA-1-dependent T-lymphocyte adhesion and lysis are independent of ICAM-1[9]. We have cloned this second ligand, designated ICAM-2, using a novel method for identifying ligands of adhesion molecules. ICAM-2 is an integral membrane protein with two immunoglobulin-like domains, whereas ICAM-1 has five[10,11]. Remarkably, ICAM-2 is much more closely related to the two most N-terminal domains of ICAM-1 (34% identity) than either ICAM-1 or ICAM-2 is to other members of the immunoglobulin superfamily, demonstrating the existence of a subfamily of immunoglobulin-like ligands that bind the same integrin receptor.

We have developed a procedure for cloning functional adhesion molecules. LFA-1 purified in the presence of Mg^{2+}, which we have found to be functionally active in binding to both ICAM-1$^+$ and ICAM-1$^-$, putative second ligand$^+$ cells (M.L.D. and T.A.S., manuscript in preparation), was coated on plastic. We used a modified version of the procedure of Aruffo and Seed for selecting complementary DNAs by expression in COS cells[12]. To test the feasibility of this procedure, COS cells were transfected with the previously cloned ICAM-1 cDNA (Fig. 1*a*). ICAM-1 was expressed on 25% of the transfected COS cells. After panning, non-adherent cells were depleted of